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PATTERNING OF BIOMOLECULES ON PLASMA-ENHANCED CHEMICAL VAPOR DEPOSITED GENERATED SURFACES (PREPRINT)

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14. ABSTRACT
Micropatterned surfaces have received extensive attention for possible applications in advanced technologies including microelectronics, microfluidics, cell growth confinement and biosensor fabrication. The latter two applications exemplify the increasing coordination between materials science and biology for future generation advanced materials. Plasma-enhanced chemical vapor deposition (PECVD) shows great promise for strengthening this aforementioned materials science and biology intersection. PECVD provides an excellent generalized platform for the incorporation of a wealth of different biomolecules and/or biologically inspired materials by way of micropatterned structures. Micropatterned substrates with site-specific binding were developed by way of self-assembled monolayer chemistry in conjunction with thin layer organic polymer deposition via PECVD. Spatial binding of biomolecules and quantum dots to PECVD patterned substrates are demonstrated.

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Micropatterned surfaces have received extensive attention for possible applications in advanced technologies including microelectronics,[1,2] microfluidics,[3,4] cell growth confinement[5,6] and biosensor fabrication.[7,8] The latter two applications exemplify the increasing coordination between materials science and biology for future generation advanced materials. Plasma-enhanced chemical vapor deposition (PECVD) shows great promise for strengthening this aforementioned materials science and biology intersection. PECVD provides an excellent generalized platform for the incorporation of a wealth of different biomolecules and/or biologically inspired materials by way of micropatterned structures.[9] Micropatterned substrates with site-specific binding were developed by way of self-assembled monolayer chemistry in conjunction with thin layer organic polymer deposition via PECVD. Spatial binding of biomolecules and quantum dots to PECVD patterned substrates are demonstrated.

Currently, surface patterning of biomolecules involves techniques such as dip pen nanolithography,[10-12] PDMS stamping,[8,13] and ink jet printing.[14] These techniques limit the number and type of biomolecules that are compatible with certain substrates. For instance, single molecule binding onto inorganic substrates generally can only be achieved by these techniques, with binding molecule selection driven by the limited surface compatibilities. PECVD shows promise to alleviate some of these inherent drawbacks associated with the previously mentioned patterning techniques. PECVD allows for a plethora of surface chemistries, as non-traditional monomers with widely varying functionalities can be plasma polymerized onto numerous substrate surfaces, including both organic and inorganic substrates.[15-17] These varying chemistries enable

surface functionality tailoring for specific molecular site binding that may not otherwise be achievable. Additional advantages of the PECVD process include solventless processing, formation of pinhole free surfaces with densely crosslinked interior morphologies, large area patterning, compatibility with a variety of substrate shapes, and room temperature operation.[18] This latter attribute is a key element for organic thin film PECVD device fabrication, as this process impinges minimal thermal load to chemically active and inert materials. Thus, minimal thermal degradation effects are encountered during deposition, which may otherwise have adverse effects on the organic components of the system. Overall, PECVD allows for substrate surfaces to be tailored for specific molecular interactions, which generates a more universal platform for device fabrication.

Micron feature size plasma polymer surface patterns on functionalized silicon wafers embody the basis of this research. Through the use of PECVD and physical masking of the substrate, high resolution micron-scale surface patterns can be deposited, with linewidth resolutions of ~ 5 μm. By plasma depositing these patterned organic thin films onto self-assembled monolayers, site-specific molecular binding can be achieved. Proof-of-concept site-specific multifunctional binding within the same substrate is demonstrated.

A high energy radio frequency-induced argon plasma, which contains a mixture of ions, radicals, electrons, and excited species, is utilized to plasma polymerize benzene and allylamine onto functionalized silicon surfaces. The plasma polymerized (pp) thin films show a densely crosslinked interior morphology, allowing for robust surface deposited

materials. More specifically, bulk pp-benzene shows less than 1% sol fraction when immersed in chloroform for an extended period. Physical masking of the surface via 1000 or 2000 mesh transmission electrom microscope (TEM) grids were employed for pattern production with 5 μm linewidth resolution. Fourier-transform infrared (FTIR) spectroscopy revealed that the plasma deposited polymers contained moieties from their native structure to a great extent, thus allowing for a high concentration of amine functional groups in the pp-allylamine surfaces (**Supplemental Material**). The surface amine concentration creates a substrate capable of site-specific molecular binding.

In this research, three distinct samples were evaluated. For the first sample, 3-aminopropyltriethoxysilane was deposited onto bare silicon and pp-benzene was patterned via PECVD onto the 3-aminopropyltriethoxysilane SAM. For the second sample, (3-mercaptopropyl)trimethoxysilane was deposited onto bare silicon and pp-benzene was patterned via PECVD onto the (3-mercaptopropyl)trimethoxysilane SAM. Finally, for the third sample, (3-mercaptopropyl)trimethoxysilane was deposited onto bare silicon and pp-allylamine was patterned via PECVD onto the (3-mercaptopropyl)trimethoxysilane SAM. The third sample was used for the multifunctional coupling evaluation experiments.

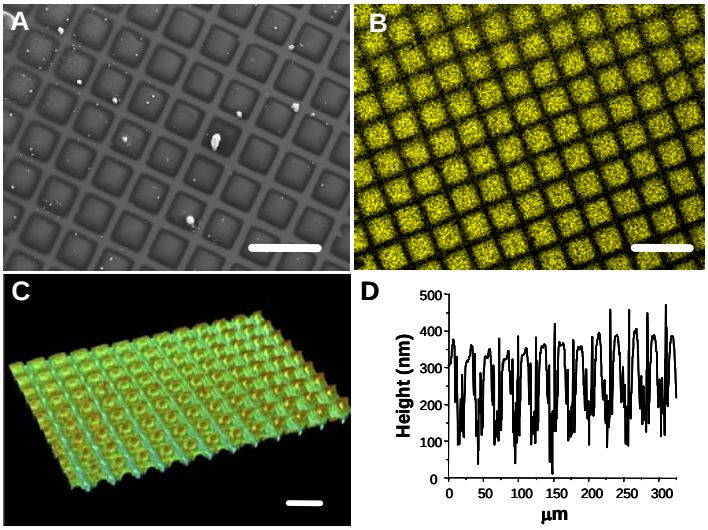

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Figure 1. Characterization of PECVD patterned surface of amine-SAM/pp-benzene. (A) SEM micrograph. (B) EDAX map of elemental carbon (in green) for pp-benzene. (C) White light interferogram displaying surface contour. (D) Height profile obtained using white light interferometry. Scale bar, 100 µm (A and B), and 50 µm (C).

Scanning electron Microscopy (SEM), elemental mapping, and white light interferometry were used to examine the pattern, composition, surface relief topology, and height profile of the patterned pp-benzene surface. By SEM, the NH_2/pp-benzene surface exhibited high definition patterned features (**Figure 1A**), while elemental mapping by energy dispersive X-ray spectroscopy (EDAX) confirmed the composition and density of the surface functionalities. The elemental maps showed emission for nitrogen, sulfur, and carbon consistent with the respective patterned regions as illustrated in the map of the NH_2-SAM/pp-benzene surface (**Figure 1B**). Topologically, the surface revealed well defined channels and discrete pp-benzene blocks with excellent structural integrity as shown in the interferogram (**Figure 1C**); however, the deposited pp-benzene islands

displayed a bowl-shaped appearance. This indentation is likely attributed to either the PECVD process, whereby the edges accumulate faster than the center producing a concave surface during deposition and/or is simply due to artifacts caused by lifting the TEM mask after deposition. Dimensionally, the blocks have a total height of 315 nm $\pm$ 21.5 and are delineated by nearly perpendicular vertical sides under the deposition conditions used (**Figure 1D**).

Given the amine- and thiol-specific surface chemistries presented; we utilized conventional 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) to couple carboxyl groups to primary amines and thiol oxidation, with a set of functionalized quantum dots, to couple to sulfhydryl groups. In the former, carboxylated quantum dots were activated by EDC and incubated with the exposed primary amines of the NH_2/pp-benzene generated surface. After coupling and multiple washes, fluorescence microscopy revealed good surface coverage of quantum dots on the patterned surface only. In the fluorescence image (**Figure 2A**), this localization within the channels of amine moieties is observed by an intense reddish fluorescence at 605 nm while minimal aggregation or adsorption to the polymerized benzene islands is observed. Alternatively, addition of carboxylate functionalized quantum dots to the NH_2/pp-benzene surface with no EDC showed a lack of fluorescence (**Supplemental Material**). Similarly, we also deposited quantum dots utilizing disulfide bond formation between the exposed thiol (-SH) groups of the PECVD patterned polybenzene surface and a thiol functionalized quantum dot under basic conditions; however, it was first necessary to derivatize the carboxylate surface of the quantum dot with an SH group. We obtained a homogeneous

surface of SH groups on the quantum dot by activating the carboxylate surface of the quantum dot via EDC and subsequent coupling with cysteamine ligands. This was confirmed by IR spectroscopy (**Supplemental Material**). Upon incubation of the cysteamine conjugated quantum dots with the thiol surface, coupling of quantum dots was observed by a characteristic blue fluorescence located along the SH channels after extensive washing as in **Figure 2B**. In addition to fluorescence, the coupled surface showed characteristic IR stretching frequencies for the conjugated quantum dots at 1720 and 1597 cm^{-1}. These single functionality surfaces of amines or thiols exhibit successful surface patterning of quantum dots via different coupling chemistries.

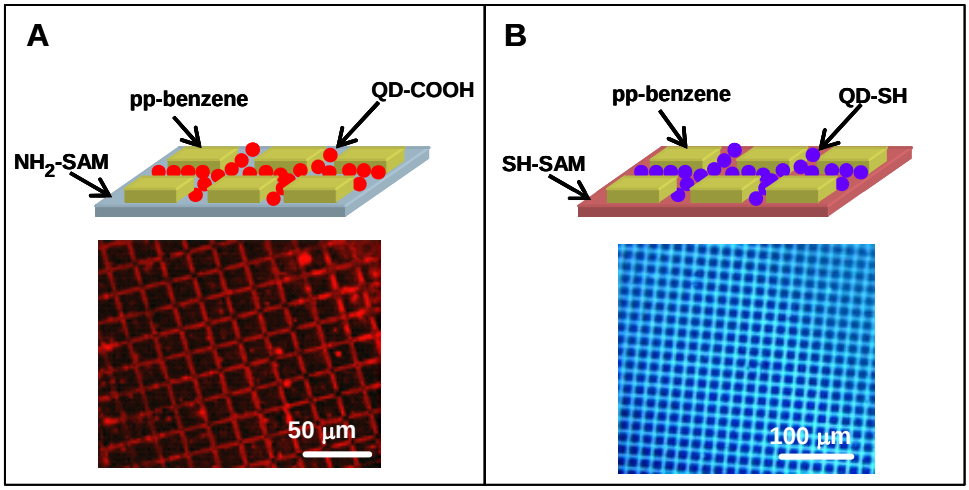

Figure 2. PECVD patterned surfaces with pp-benzene and variable surface chemistries (NH_2 or SH). (A) Fluorescence image of carboxylate functionalized quantum dots coupled to NH_2-SAM/pp-benzene by EDC (excitation ~ 400 nm). (B) Fluorescence image of quantum dots conjugated with cysteamine and coupled to SH-SAM/pp-benzene by disulfide formation.

Moreover, we have incorporated these parallel coupling techniques with an SH-SAM/pp-allylamine (NH_2) mixed functionality surface for the patterning of quantum dots and

proteins. Recently, nanoscale patterning of green fluorescent protein (GFP) was demonstrated using electron beam lithography and modification to GFP with excellent resolution.[19] Comparatively, with our mixed functionality surface, we patterned GFP by targeting the PECVD pp-allylamine NH_2 exposed islands. GFP was chosen because of its strong fluorescence upon excitation of its internal fluorophore (quantum yield is similar to that of quantum dots), similar excitation wavelength profile (395 nm) to orange fluorescent quantum dots, sensitivity of emission to changes in protein structure and folding, protein stability, and abundance of amine and carboxyl functionalities.[19,20]

After coupling, we obtained highly patterned GFP within the pp-allylamine NH_2 regions as observed by intense green fluorescence (**Figure 3**) with no apparent loss to the fluorescence as a result of being constrained along the surface, thus confirming that GFP retains it native structure. This was followed by selective oxidation of the sulfhydryl groups of cysteamine conjugated orange fluorescent quantum dots with the SH groups of the PECVD generated surface to a disulfide linkage. Coupling of quantum dots within the SH grid channels was clearly seen by fluorescence microscopy and complemented by the fluorescence of GFP as a multi-color fluorescence pattern. Remarkably, there is only a minimal amount of nonspecific coupling of orange quantum dots to the patterned GFP surfaces, thus highlighting the specificity of parallel couplings.

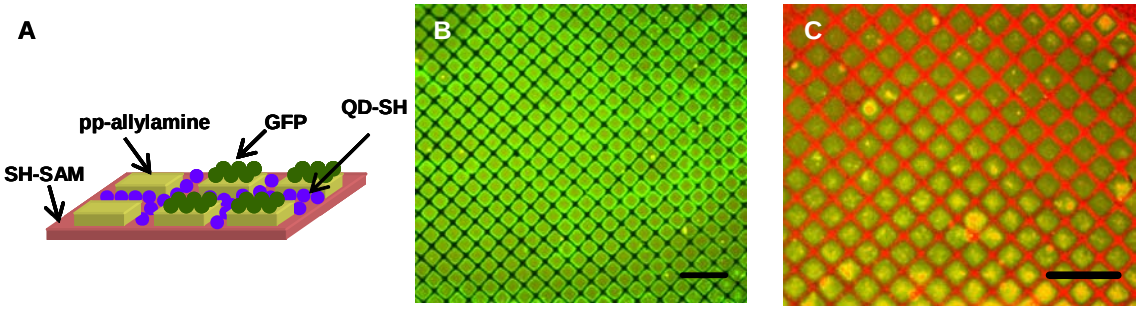

Figure 3. Coupling to SH-SAM/pp-allylamine multifunctionality surface. (A) Illustration depicts composition and selective binding of GFP and QDs. (B) Fluorescence of coupled GFP to pp-allylamine patterns by EDC (excitation filter 400-440 nm). (C) Dual-color fluorescence of GFP coupled to the amine groups on the patterned pp-allylamine and QDs coupled to the thiol-SAM (excitation ~ 400 nm). Scale bar, 100 μm.

Quantum dots and proteins represent a fraction of biomolecular materials that can be patterned using PECVD generated surfaces. Others potentially include viruses, antibodies, cells, and enzymes; all of which contain amine, carboxylate, and/or thiol groups. Of interest is the highly specialized biological activity/recognition they impart to a surface that is necessary for the fabrication of biosensors/devices; but also a means to improve performance, enhance stability, and isolate biomolecules from products.[20] For example, the immobilization of enzymes can offer such improvements when constrained on self-assembled monolayers,[21] nanoparticle/colloidal surfaces,[22,23] and sol-gel surfaces.[24] We also examined the effect of enzyme immobilization onto the PECVD patterned substrate. Horse radish peroxidase (HRP), a commonly used enzyme, was immobilized on the NH_2-SAM/pp-benzene patterned surface. The activity immobilized enzyme was compared to that of the free enzyme. As shown in Figure 4, the immobilized enzyme exhibited comparable activity to the free enzyme in solution as noted by the

absorbance peak at 405 nm indicative of the substrate product. Additionally, the substrate with the immobilized enzyme could be used for twice over before the enzyme lost ~90% of its original activity.

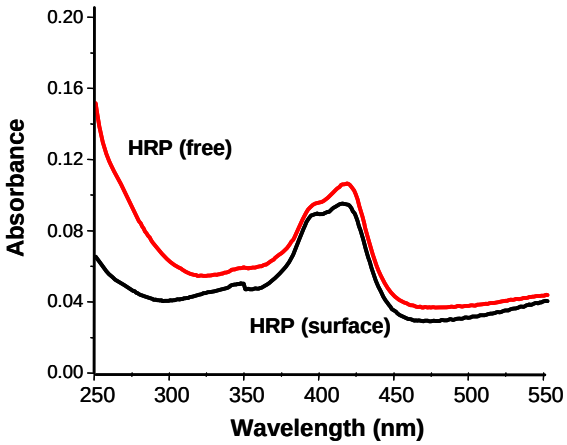

Figure 4. Enzymatic activity of horseradish peroxidase (HRP) coupled to the NH_2-SAM/pp-benzene patterned surface. The UV-Vis spectra of product after incubation of the substrate, ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) with either free or immobilized HRP.

PECVD provides an effective platform for precisely tailoring surfaces properties such as hydrophobicity, type and density of functional groups, and for the incorporation of a diverse array of biomolecules by independently coupling different molecules selectively based on compatible surface chemistries. These surfaces are also amenable to various kinds of inorganic nanoparticles. The incorporation of a second functionality adds the capability to pattern multiple biological targets essentially in a one-pot mixture.

Experimental

***Self-Assembled Monolayer Deposition*:** Prior to self-assembled monolayer (SAM) deposition, all silicon wafers were surface cleaned via piranha etch (3:1 sulfuric

acid:hydrogen peroxide) for 1 hr. SAMs were deposited via a solution method.[17] Thiol-terminated SAM was deposited by immersing a clean silicon wafer into a toluene solution with 1.5 wt% 3-mercaptopropyltrimethoxysilane and 0.5 wt% butylamine as catalyst. Amine-terminated SAM was deposited by immersing a clean silicon wafer into a toluene solution with 1.5 wt% 3-aminopropyltriethoxysilane. In both solutions, the silicon wafer was allowed to react for 45 min. After reaction was complete, the silicon wafers were removed from the toluene solutions and washed with pure toluene and acetone and blown dry with dry nitrogen. The deposited wafers were then annealed under vacuum at room temperature for three days. SAM deposition was checked via water contact angle goniometry. Measured advancing contact angles were 45 ± 2° for amine-terminated SAM and 44±3° for thiol-terminated SAM, which is in good agreement with literature values.

***Plasma-Enhanced Chemical Vapor Deposition of Surface Gratings*:** The plasma reactor system utilized for the thin film polymer deposition is described elsewhere.(ref) The vacuum in the 10 cm reactor chamber was maintained at 0.2 – 0.05 Torr, while 50 – 100 standard cm^3/min of 99.999% argon carrier gas flowed through capacitively coupled electrode plates, with a primary radio frequency discharge at 13.56 MHz. Monomer vapors were introduced into the reactor chamber in the center of the plasma zone between the coupled electrode plates. The deposition substrate was then placed 8 cm further downstream from the plasma zone inlet. Benzene and allylamine flow rates were controlled by a manually adjusted high-accuracy metering valve. The monomer flow rates were controlled in the range of 0.5 – 5 cm^3/min. Deposition times were 6 minutes for pp-benzene and 17 min for pp-allylamine. High definition surface gratings were

formed with 1000 or 2000 mesh copper TEM grids that were adhered to the surface of the substrate prior to the plasma polymer deposition.

***Cysteamine conjugation**:* 2 μL of Fort Orange (Lot #AW40422ATF) or Lake Placid Blue (Lot #AW40114CTL) CdSe/ZnS Evitag-carboxyl quantum dots (0.25 mg/mL, Evident Technologies) was mixed with 2 μL of EDC/sulfo-NHS and an excess of 0.1 M cysteamine (Aldrich, 2 x 10^{-5} moles) in a microfuge tube and incubated for 30 minutes. Using a Microcon YM-10 spin filter with regenerated cellulose 10,000 MWCO membrane (Amicon), 100 μL of the cysteamine conjugated quantum dots were purified at 4000 rpm using a Fisher Microfuge by concentrating down to 100 μL, diluting to 400 μL with water, and concentrating to a final volume of 50 μL.

***Patterned PECVD surface coupling (NH_2 SAM/pp-benzene) or (SH SAM/pp-benzene)**:* 1 μL of carboxyl quantum dots was added to NH_2/pp-benzene surface followed by 1 μL of EDC/sulfo-NHS. After 20 minutes, this was removed from surface and washed repeatedly with 3 μL of doubly deionized water 3x. Similarly, for the SH SAM/pp-benzene surface, 1 μL of cysteamine conjugated quantum dots and 2 μL of 0.1 M Tris buffer pH 9.2 was added for 20 minutes and washed.

Mixed SH/pp-allylamine surface coupling: 1 μL of green fluorescent protein at 23 μM (previously expressed and purified) was added to surface with 1 μL of EDC/sulfo-NHS and incubated for 20 minutes. GFP was removed and the surface was washed 3x with water. This was followed by addition of 1 μL of cysteamine conjugated Fort Orange quantum dots and 2 μL of 0.1 M Tris buffer pH 9.2. Again, the quantum dots were incubated for 20 minutes and washed.

Enzyme Assay: The activity of immobilized and free horseradish peroxidase (Sigma-Aldrich, St Louis, MO) was monitored using 2,2'-Azino-bis[3-Ethylbenzthiazoline-6-Sulfonic acid] (ABTS) as substrate. ABTS is a widely used substrate for peroxidase and produces a green colored product that can be read spectrophotometrically at 405 nm.

Characterization*:* Fluorescence images were obtained on an Olympus BX51 epifluorescence microscope equipped with CCD camera and appropriate excitation filters, while FT-IR was performed on a Perkin Elmer Auto-Image IR microscope using a double sided polished silicon wafer. SEM was done by mounting silicon wafer on an SEM puck on a Phillips XL FEG eSEM operating at 10 kV and a working distance of 10 mm. White light interferometry was performed on a Veeco WYKO NT1100 optical profiling system.

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